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Estimating the Instability Parameters of Plasmid-Bearing Cells. I. Chemostat Culture

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What determines the stability of plasmid-bearing cells in natural and laboratory conditions? In order to answer this question in a quantitative manner, we need tools allowing the estimation of parameters governing plasmid loss in different environments. In the present work, we have developed two methods for the estimation of the instability parameters of plasmid-bearing cells growing in chemostat. These instability parameters are: (i) selection coefficient (or cost of the plasmid) α and (ii) the probability of plasmid loss at cell division τ_0. We have found that generally selection coefficient α changes during elimination of plasmid-bearing cells due to changes in substrate concentration; hence, methods which assume constancy of α are intrinsically imprecise. Instead, one can estimate selection coefficient at the beginning and the end of cultivation when the substrate concentration is approximately constant. Applying developed techniques to two sets of experimental data, we have found that (i) the cost of the plasmid pBR322 depended on the dilution rate in chemostat and was higher at low dilutions; (ii) high levels of plasmid gene expression led to a high cost of the plasmid pPHL-7; (iii) the probability of plasmid loss was lower at high levels of plasmid gene expression and independent of the dilution rate. We have also discussed the application of our results to understanding the basic biology of bacterial plasmids.

Introduction

What are plasmids? Plasmids are extrachromosomal DNA molecules capable of stable maintenance in microbial populations. Being an important part of bacterial evolution (Levin & Bergstrom, 2000), plasmids are the major cause of the widely disseminated antibiotic resistance among natural microorganisms (Levin *et al.*, 1997). Understanding the causes of stability (or instability) of plasmid-bearing cells in different environmental conditions is the first step in the fight against resistant microbes.

Why are plasmids unstable in some laboratory conditions whereas in natural environments they seem to be maintained without any significant effort? How can we compare different plasmids based on their stability? Knowledge of the molecular structure and the mechanisms of copy number control and inheritance of plasmids rarely can help one to understand whether plasmids will persist in a host population. Only measurements on the population level and estimation of the rate at which plasmid-bearing

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cells are substituted by plasmid-free counterparts can answer this question.

Possible causes of instability. During growth of bacteria plasmid-free variants arise in the initially homogeneous plasmid-bearing cell population basically in two ways. First, each plasmid-bearing cell has a probability to give rise to a plasmid-free cell at cell division (this depends on the mechanisms of plasmid distribution between daughter cells, plasmid copy number at the cell division, the presence of multimer resolution loci, etc.). Usually, such probability is very low for natural plasmids (about $<10^{-7}$) whereas recombinant plasmids (i.e. genetically modified) may segregate with a higher probability (10^{-3}–10^{-5}, Summers, 1996). Several hypotheses have been put forward to explain this tremendous difference: impaired copy number control (Paulsson & Ehrenberg, 1998), the absence/impairment of the multimer resolution genes (Summers & Sherratt, 1984), and random distribution of plasmid among daughter cells for low copy number plasmids (Nordstrom *et al*., 1984).

Second, it was experimentally found that plasmid-bearing cells usually have a lower maximum specific growth rate than their plasmid-free counterparts (Zund & Lebek, 1980; Bentley *et al*., 1990; Andersson & Levin, 1999, and references therein) and once a plasmid-free cell has arisen, it outcompetes its plasmid-bearing counterparts very rapidly. Since most recombinant plasmids are not conjugative (not capable of self-transfer to other plasmid-free cells), if a cell has lost a plasmid, there is no way for the cell to acquire it again. Thus, a segregation of plasmids at cell division and the difference in the growth rates of plasmid-free and plasmid-bearing subpopulations determine the rate at which plasmids are lost during prolonged cultivation.

How can we compare plasmids based on their stability? There are at least two methods. One is just to observe which plasmid is lost faster and then to claim that this plasmid is less stable. This approach is purely qualitative; it also does not inform about the causes of such instability. On the other hand, we may construct simple models governing the process of plasmid loss, and, using experimental data, we can estimate key parameters determining the stability of plasmid-bearing cells. A similar quantitative approach has been applied for kinetic characterization of bacterial strains grown in a given environment based on their maximal growth rate μ_{max} and half-saturation constant K_S (Monod, 1949, Pirt, 1975, Lendenmann *et al*., 2000).

To compare population stability of different plasmids transformed in a particular bacterial strain or stability of a given plasmid-bearing population in different environments, a simple mathematical model can be constructed. The model assumes that a bacterial population consists of two parts: plasmid-bearing cells X^+ and their plasmid-free counterparts X^- (Stewart & Levin, 1977). Plasmid-bearing cells can lose the plasmid at cell division; this process is assumed to be proportional to the specific growth rate of the plasmid-bearing subpopulation, $\mu^+(S)$. The mathematical model for the case of growth of bacteria in a chemostat culture can be written as a system of differential equations:

$$\frac{dX^+(t)}{dt} = (\mu^+(S) - D)X^+(t) - \tau_0\mu^+(S)X^+(t),$$

$$\frac{dX^-(t)}{dt} = (\mu^-(S) - D)X^-(t) + \tau_0\mu^+(S)X^+(t),$$

$$\frac{dS(t)}{dt} = D(S_0 - S) - \frac{\mu^+(S)X^+(t)}{y^+} - \frac{\mu^-(S)X^-(t)}{y^-}, \quad (1)$$

where $\mu^+(S)$, $\mu^-(S)$ denote specific growth rates of each subpopulation; D is a specific dilution rate in chemostat [if $D = 0$ then system (1) describes the growth of bacteria in batch culture]; τ_0 is proportional to the probability of plasmid loss at cell division,§ S and S_0 are growth-limiting substrate concentrations in vessel and feed reservoirs, respectively; y^+ and y^- are yield coefficients for plasmid-bearing cells and their plasmid-free counterparts (Pirt, 1975; Bailey *et al*., 1986). Growth rates of

§ $\tau_0 \approx \theta/2$, where θ is the probability that a plasmid-bearing cell gives rise to a plasmid-free cell at cell division (Bailey *et al*., 1986; Dunn *et al*., 1995; Boe & Rasmussen, 1996).

plasmid-bearing and plasmid-free cell populations are assumed in the form suggested by Monod (Monod, 1949; Pirt, 1975):

$$\mu^{\pm}(S) = \frac{\mu^{\pm}_{max} S}{K^{\pm}_S + S}, \tag{2}$$

where μ_{max} is a maximum specific growth rate of the population, and K_S is a half-saturation constant (which equals the concentration of substrate at which the population grows at half its maximum rate). The "cost" that plasmids impose on their host cells can be measured by the selection coefficient (also called selection advantage of plasmid-free cells) that is a relative growth rate difference

$$\alpha(S) = 1 - \frac{\mu^{+}(S)}{\mu^{-}(S)}. \tag{3}$$

(Note that $\alpha = 0$ if $\mu^{+} = \mu^{-}$ and $\alpha = 1$ if $\mu^{+} = 0$.) Therefore, two parameters α and τ_0 according to this simple model represent the basic characteristics of plasmid-bearing cells (or just the plasmid possessed by bacteria) as, for instance, μ_{max} and K_S are general characteristics of microorganisms in a given environment (Pirt, 1975).‖

The general purpose of this paper is to review the existing methods applied to estimate the parameters α and τ_0 from the population dynamics of plasmid-bearing cells in chemostat and to discuss advantages and shortcomings of these methods. We do so by analysing the dynamics of model (1) and comparing how different approximations fit to the original dynamics. Finally, we apply one previously suggested method analysed by Davidson *et al.* (1990) and two methods developed in this paper to two sets of experimental data and discuss what knowledge we can infer from such estimates and what the estimated values tell us about the biology of bacterial plasmids.

‖ Other parameters can also be used to characterize the stability of plasmid-bearing cells: $R = \tau_0 \mu^{+}(S)$ is the rate of plasmid loss, $C = \mu^{-}/\mu^{+}$ is the ratio of generation times, etc. (Cooper *et al.*, 1987; Caulcott *et al.*, 1987; Proctor, 1994; Dunn *et al.*, 1995; Boe & Rasmussen, 1996).

Analysis and Results

THE DYNAMICS OF PLASMID-BEARING CELLS IN CHEMOSTAT

Mathematical model (1) describes elimination of plasmid-bearing cells from bacterial population grown in chemostat; the model involves several parameters that determine the rate at which plasmid-bearing cells are substituted by their plasmid-free counterparts. The elimination of plasmid-bearing cells in chemostat can be subdivided into three phases.¶

Initially, there are no or only few plasmid-free cells in the population. If there are no plasmid-free cells in the population [$X^{-}(0) = \tau_0 = 0$] then a steady state exists which is determined by classic equations (Pirt, 1975):

$$\mu^{+}(\hat{S}^{+}) = D,$$

$$\hat{S}^{+} = \frac{K^{+}_S D}{\mu^{+}_{max} - D},$$

$$\hat{X}^{+} = y^{+}(S_0 - \hat{S}^{+}). \tag{4}$$

However, since in the model $\tau_0 > 0$ the relative number of plasmid-free cells increases with time (at $\alpha \geqslant 0$); yet because plasmid-bearing cells are the predominant part of the population consuming the substrate, the substrate concentration does not change significantly at the beginning of cultivation [first 15 hr in Fig. 1(B) while $n^{+} = X^{+}/(X^{+} + X^{-}) > 95\%$]. Thus, during this phase the substrate concentration is approximately equal to that at the steady state (4). Later, during the second phase, the fraction of plasmid-bearing cells declines [$10\% \leqslant n^{+} \leqslant 95\%$, Fig. 1(A)].

Finally, during the third phase the opposite to the first phase dynamics is observed. There are only few plasmid-bearing cells, and when all of them are eliminated (at $t \to \infty$) the true steady state is achieved:

$$\mu^{-}(\hat{S}^{-}) = D, \qquad \hat{S}^{-} = \frac{K^{-}_S D}{\mu^{-}_{max} - D},$$

$$\hat{X}^{-} = y^{-}(S_0 - \hat{S}^{-}). \tag{5}$$

¶ We do not consider the start-up phase assuming that the culture is at the steady state (4) at $t = 0$.

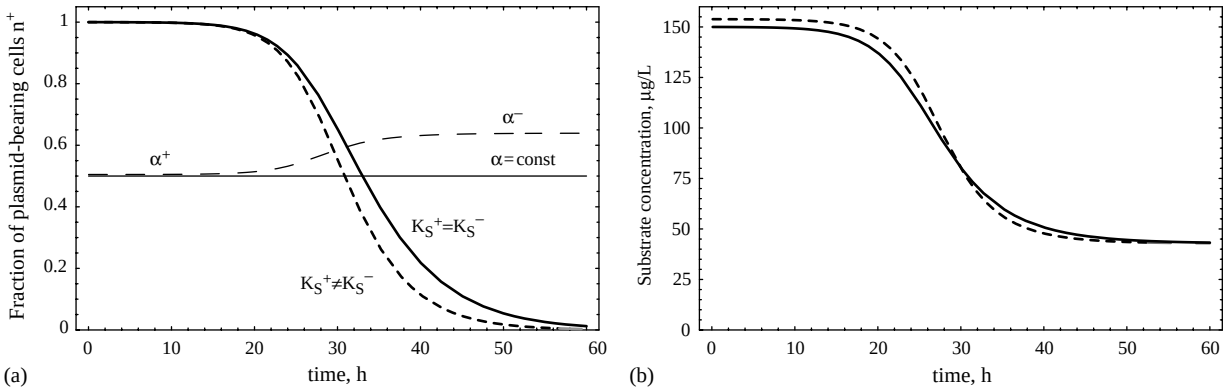

FIG. 1. The dynamics of plasmid-bearing cells in chemostat: a mathematical model. We have generated the curves by a numerical solution of the basic model (1) for two sets of parameters. Panel A shows the change in the fraction of plasmid-bearing cells in the population (thick lines) and selection coefficient at the beginning (α^+) and the end (α^-) of cultivation (thin lines). Panel B shows the change in the remaining concentration of the limiting growth of bacteria substrate S. "—": $K_S^+ = K_S^- = 100\,\mu g/l^{-1}$, $\mu_{max}^+ = 0.5\,hr^{-1}$, $\mu_{max}^- = 1\,hr^{-1}$; "- - -": $K_S^+ = 333\,\mu g/l^{-1}$, $\mu_{max}^+ = 0.95\,hr^{-1}$, $K_S^- = 100\,\mu g/l^{-1}$, $\mu_{max}^- = 1\,hr^{-1}$. Other parameters: $D = 0.3\,hr^{-1}$, $S_0 = 1\ g/L$, $y^+ = y^- = 0.5$, $\tau_0 = 10^{-4}$, $X^-(0) = 0$, and the initial density of plasmid-bearing cells and substrate is at the steady state given by eqn (4).

When $0 < n^+ < 10\%$ the substrate concentration again remains approximately constant [Fig. 1(B) at $t > 45$ hr] and is approximately equal to that at the steady state defined in eqn (5). Thus, the three phases during which the elimination of plasmid-free cells occurs are: (1) most cells in the population are plasmid-bearing [$S(t) \approx \hat{S}^+$], (2) there is an approximately equal number of both cell types [$S^- < S(t) < S^+$], and (3) most cells are plasmid-free [$S(t) \approx \hat{S}^-$]. Moreover, in order to adequately estimate the instability parameters α and τ_0, one needs to account for the fact that the substrate concentration changes during competition of plasmid-free and plasmid-bearing cells [Fig. 1(B)].

The last observation is particularly important in light of the fact that selection coefficient defined in eqn (3) generally depends on the substrate concentration (except in the rare case when half-saturation constants for plasmid-free and plasmid-bearing cells are identical) and therefore, changes with changes in substrate concentration [Fig. 1(A), thin dashed lines]. This fact has been largely ignored in all methods for the estimation of instability parameters α and τ_0 known to us (Lenski & Bouma, 1987; Cooper *et al.*, 1987; Davidson *et al.*, 1990; Duetz & van Andel, 1991; Proctor, 1994), even though it is not known *a priori* that α does not depend on the growth conditions (i.e. substrate concentration) and needs to be verified in each particular case (see below). We, therefore, are going to derive approximations of the basic model (1) which allow us to estimate the selection coefficient in the beginning of the cultivation process [we call it $\alpha^+ = \alpha(\hat{S}^+)$ because most cells in the population at this time are plasmid-bearing cells] and during the elimination of plasmid-bearing cells [$\alpha^- = \alpha(\hat{S}^-)$, i.e. when most cells are plasmid-free]. We also will derive an exact approximation for model (1) if the assumption $\alpha = const$ is valid.

GENERAL CASE: $\alpha \neq CONST$

Selection coefficient defined in eqn (3) generally depends on both maximum specific growth rates and half-saturation constants of plasmid-free and plasmid-bearing cells. When estimated in batch culture where substrate concentration is high, selection coefficient is determined mostly by the maximum specific growth rates. In contrast, in chemostat both parameters may be important. The only exception is when half-saturation constants for plasmid-bearing and plasmid-free cells are equal; then $\alpha = 1 - \mu_{max}^+/\mu_{max}^- = const$. Unfortunately, there is no good data on whether $K_S^+ = K_S^-$ for most plasmid–host associations. If the half-saturation constants are different for plasmid-free cells and

their plasmid-bearing counterparts, selection coefficient is not constant, and there is no an easy way to estimate how rapidly it changes (increases) during elimination of plasmid-bearing cells [Fig. 1(A)]. Hence, we suggest to use approximations of the growth of bacteria in chemostat when substrate concentration is approximately constant [given by eqns (4) and (5)] to estimate the selection coefficient at the beginning (α^+) and the end of cultivation (α^-).

We define two useful characteristics

$$z(t) = \frac{X^-(t)}{X^+(t)},$$

$$n^+(t) = \frac{X^+(t)}{X^+(t) + X^-(t)} = \frac{1}{1 + z(t)}, \qquad (6)$$

where z is a ratio of the number of plasmid-free to plasmid-bearing cells, and n^+ is a fraction of plasmid-bearing cells in the microbe population. Then using the definition of z, we can rewrite the first two equations of model (1) in two forms:

$$\frac{dz}{dt} = \mu^+(S)\left[\frac{\alpha(S)}{1 - \alpha(S)}z + \tau_0(1 + z)\right], \qquad (7)$$

$$\frac{dz}{dt} = \mu^-(S)[\alpha(S)z + \tau_0(1 - \alpha(S))(1 + z)]. \qquad (8)$$

Note that in general case neither of these equations can be solved analytically because of the dependence of α, μ^+, and μ^- on the substrate concentration.

Estimating Selection Coefficient α^-

Selection coefficient of plasmid-bearing cells can be determined if we assume that the substrate concentration is at the steady state given by eqn (5). Note that this is true only at the end of competition, i.e. when $z \gg 1$. Using the approximation $\mu^-(S) \approx D$, we can rewrite eqn (8) in the form

$$\frac{dz}{dt} \approx \alpha^- Dz + \tau_0 D(1 - \alpha^-)(1 + z), \qquad (9)$$

and find its solution by direct integration:

$$z(g) = z(0)2^{(\tau_0(1-\alpha^-)+\alpha^-)g} + \frac{\tau_0}{\tau_0 + \alpha^-/(1 - \alpha^-)}\left(2^{(\tau_0(1-\alpha^-)+\alpha^-)g} - 1\right), \qquad (10)$$

where $g = Dt/\ln 2$ is the number of generations in chemostat. Since we assume that $z \gg 1$, we can neglect the second term in eqn (10) and using linear regression we find an estimate for α^- (at $\tau_0 \ll \alpha^-$)

$$\ln z(g) \approx \ln C + \alpha^- g \ln 2. \qquad (11)$$

(Note that since we estimate parameter $\tau_0(1 - \alpha^-) + \alpha^-$, the estimated value is an upper bound for α^-.) A similar expression has been suggested by several other researchers for estimation of selection coefficient in chemostat and batch cultures (Cooper *et al.*, 1987; Brilkov *et al.*, 1990; Proctor, 1994; Boe & Rasmussen, 1996). Therefore, approximating experimental data by solution (11) when $z \gg 1$ one can estimate the cost of the plasmid α^- at the end of competition between plasmid-bearing cells and their plasmid-free counterparts.** Note that this estimate is the maximal cost of the plasmid at given conditions (α^+ gives a lower bound estimate).

Estimating a Probability of Plasmid Loss τ_0 and Selection Coefficient α^+

Similar to the previous case by assuming that the substrate concentration at the beginning of cultivation is at the steady state given by eqn (4), we find using eqn (8) that the ratio z obeys the equation (at $z(0) = 0$)

$$\ln z(g) \approx \ln\left[\frac{\tau_0(1 - \alpha^+)}{\tau_0(1 - \alpha^+) + \alpha^+}\right] + \ln\left[2^{(\tau_0 + \alpha^+/(1-\alpha^+))g} - 1\right]. \qquad (12)$$

** This approximation, however, can be used even at $n^+ \sim 20\%$ if the log-transformed data represent a straight line.

Two points need to be emphasized. First, the probability of plasmid loss τ_0 can be estimated only if the initial fraction of plasmid-free cells is sufficiently small [$z(0) \approx 0$], and all plasmid-free cells arise only through segregation (loss at cell division) and following competition with plasmid-bearing counterparts. Otherwise the estimation is not precise (Duetz *et al.*, 1991, not shown). Second, in order to estimate α^+ and τ_0, one needs to apply nonlinear fitting techniques to fit the theoretical curve (12) to the experimental data.

PARTICULAR CASE: $\alpha = CONST$

Making the assumption $\alpha = const$ (which probably is not valid for at least some examined systems (Godwin & Slater, 1979; Duetz & van Andel, 1991)) simplifies the analysis of the basic model and allows to find its explicit solution. The general approach previously applied to estimate parameters α and τ_0 at the approximation $\alpha = const$ has been done by assuming a constant steady state in chemostat with $\mu^+(S) = D$ (Davidson *et al.*, 1990; Dunn *et al.*, 1995). Rewriting eqn (7) by replacing $z(t)$ with $n^+(t)$ we obtain

$$\frac{dn^+}{dt} = -\mu^+(S)n^+\left[\frac{\alpha(S)}{1-\alpha(S)}(1-n^+) + \tau_0\right]. \quad (13)$$

Assuming that $\mu^+(S) = D$ and $\alpha = const$ we find its explicit solution

$$n^+(g) = \frac{n^+(0)(\tau_0(1-\alpha)+\alpha)}{\alpha n^+(0) + (\tau_0(1-\alpha) + (1-n^+(0))\alpha)2^{(\alpha/(1-\alpha)+\tau_0)g}}, \quad (14)$$

where $g = \mu^+ t/\ln 2 = Dt/\ln 2$ (Walmsley *et al.*, 1983; Lenski & Bouma, 1987; Davidson *et al.*, 1990; Proctor, 1994; Dunn *et al.*, 1995).

We suggest a more elegant approach to estimate parameters α and τ_0 if $\alpha = const$; this method has been previously developed at the approximation $\tau_0 = 0$ by Duetz *et al.* (1991). Their major observation [taken from Cooper *et al.*, 1987] was that after a short phase of expansion of plasmid-bearing cells in the chemostat, the fraction of plasmid-bearing cells obeys the equality

$$\mu^+(S)n^+ + \mu^-(S)(1-n^+) = D. \quad (15)$$

The growth rate of plasmid-bearing cells can be then determined explicitly:

$$\mu^+(S) = \frac{D(1-\alpha)}{1-\alpha n^+}. \quad (16)$$

Replacing $\mu^+(S)$ in eqn (13) with eqn (16), assuming $\alpha = const$, and integrating we obtain

$$\left[\frac{\alpha(1-n^+(g)) + \tau_0(1-\alpha)}{\alpha(1-n^+(0)) + \tau_0(1-\alpha)}\right]^{(1-\tau_0)(1-\alpha)} = \left(\frac{n^+(g)}{n^+(0)}\right)2^{(\alpha+\tau_0(1-\alpha))g}, \quad (17)$$

where $g = Dt/\ln 2$. By fitting solution (17) to the experimental data, one can estimate the parameters α and τ_0.

A Comparison of Different Methods — Advantages and Shortcomings

In order to evaluate the quality of methods developed in this paper and the method suggested previously (Walmsley *et al.*, 1983; Lenski & Bouma, 1987; Davidson *et al.*, 1990; Dunn *et al.*, 1995), we have generated two sets of data from numerical solutions of the basic mathematical model [for $K_S^+ = K_S^-$ and $K_S^+ \neq K_S^-$; see Fig. 1(A)] and fitted these data by (i) previously suggested solution (14), (ii) solution (17) found in this paper at the approximation $\alpha = const$, and (iii) solutions (11) and (12) found when $\alpha \neq const$. The results of the fitting are summarized in Table 1.

$\alpha = const$. Apparently, both methods developed in this paper find plasmid parameters reasonably well for the case when selection coefficient is independent of the substrate concentration (i.e. when $K_S^+ = K_S^-$) (Methods 2 and 3 in the first row in Table 1). A method of nonlinear fitting analysed by Davidson *et al.* (1990) fails to obtain relatively good estimates (Method 1 in Table 1). This fact is a direct consequence of the approximation used at the derivation of the model solution (14), that is

TABLE 1
Estimation of the plasmid instability parameters: a mathematical model

Data set	Method 1*		Method 2†		Method 3‡		
	α	$\tau_0(10^{-4})$	α	$\tau_0,(10^{-4})$	α^+	α^-	$\tau_0,(10^{-4})$
$K_S^+ = K_S^-$§	0.40 ± 0.08	8.0 ± 1.6	0.50 ± 0.01	1.00 ± 0.08	0.49 ± 0.01	0.51 ± 0.01	0.87 ± 0.10
$K_S^+ \neq K_S^-$‖	0.46 ± 0.05	3.5 ± 0.6	0.58 ± 0.02	0.10 ± 0.01	0.50 ± 0.01	0.66 ± 0.01	0.52 ± 0.08

*† Fitting by analytical solutions (14) and (17), respectively.
‡ Fitting of five initial and four last points by solutions (12) and (11), respectively. Functions implemented in *Mathematica* have been used for fitting (Wolfram, 1990).
§ $K_S^+ = K_S^-$ ($\alpha = 0.5$, $\tau_0 = 10^{-4}$).
‖ $K_S^+ \neq K_S^-$ ($\alpha^+ \approx 0.505$, $\alpha^- \approx 0.64$, $\tau_0 = 10^{-4}$); other parameters are the same as in Fig. 1. For fitting 15 equally distributed points have been used.

$\mu^+(S) = D$. When the number of plasmid-free cells increases, the substrate concentration falls, and this decrease is high for large α's. Since this change is not reflected in approximation (14), it is the major reason why this method gives incorrect estimates for both parameters. If, however, selection coefficient is relatively small ($\alpha < 0.1$) this method gives estimates relatively close to the real parameter values (not shown). In contrast, the exact solution (17) gives the best estimates for the parameters used to generate data sets independently of the selection coefficient used.

Fitting the data by Method 3 that uses only partial data also gives estimates which are in a fairly good agreement with the known parameter values at $\alpha = const$. Such consistency, however, should not be viewed as a sign of an appropriate method. The general problem when such methods are used (similar approaches have been undertaken by Cooper *et al.*, 1987; Caulcott *et al.*, 1987; Brilkov *et al.*, 1990; Proctor, 1994) is that the number of points chosen for regression needs to be defined by the researcher and, therefore, is biased. Moreover, since only a part of the data is used to estimate α and τ_0 such methods are generally less accurate than nonlinear methods (give larger errors for estimated parameters) that employ the whole data sets (see a similar discussion in Davidson *et al.*, 1990).

$\alpha \neq const$. This case demonstrates incompetence of the methods derived at the assumption $\alpha = const$ in finding correct estimates for the instability parameters if $K_S^+ \neq K_S^-$. Interestingly, Method 2 (Table 1) gives an estimate for α that is approximately an average of α^+ and α^- even though estimated τ_0 is of order of magnitude lower than a true value. On the contrary, parameter values found with the use of Method 3 are the closest to the parameters used in the model (i.e. α^+, α^- and τ_0); the found estimates, however, are sensitive to the number of points chosen for the fitting. This is reflected in the fact that estimated τ_0 is lower than a true value and has a large standard error (compare with Method 2, see Table 1).

Summary

The results of the analysis clearly demonstrate that estimation of the instability parameters of plasmid-bearing cells even growing in approximately constant environmental conditions (i.e. in chemostat) is not simple. On the one hand, we do not actually know whether selection coefficient depends on substrate concentration and, therefore, which method should be applied to infer model parameters from experimental data [both curves in Fig. 1(A) look very much alike]. The only case when selection coefficient is constant is when $K_S^- = K_S^+$. This problem can be overcome by using Method 3 (see above) which makes no assumption regarding selection coefficient. On the other hand, if Method 3 is applied to estimate α and τ_0, the found estimates may greatly depend on the number of points used to fit the model to the data.

One possible way to apply the techniques developed in this paper is as follows. First, estimate parameters α^+, α^-, and τ_0 using Method 3 [eqns (11) and (12)]. Then if $\alpha^+ \approx \alpha^-$, using Method 2 [eqn (17)] estimate the average α and τ_0 . Unfortunately, this algorithm does not work perfectly well; for instance, we find that even

when $\alpha^+ \approx \alpha^-$, the average selection coefficient may differ significantly from either α^+ or α^- (see the next section). A possible cause of such dissimilarity in estimated parameters is probably due to bias of Method 3 in choosing the number of points for the fitting.

APPLICATION OF THE METHODS TO EXPERIMENTAL DATA

As an example, using three discussed methods, we have estimated the instability parameters of plasmid-bearing cells growing in chemostat on different sugars as the only source of carbon and energy and at different dilutions rates (Wouters *et al.*, 1980; Popova *et al.*, 1992). Wouters *et al.* have investigated how rapidly plasmid-bearing cells (plasmid pBR322) are eliminated during cultivation in chemostat with the limitation of the growth by glucose at two different dilution rates ($D = 0.1$ and $0.3\,\mathrm{hr}^{-1}$, see Fig. 2). Popova *et al.* have analysed the stability of plasmid-bearing cells (plasmid pPHL-7) in chemostat limited by glucose or glycerol [the expression level of the *lux* operon cloned on the plasmid pPHL-7 depends on the substrate used for the growth: glycerol promotes the expression (glowing), whereas glucose represses it]. Estimated by three different methods, parameters α and τ_0 are given in Table 2; the actual data and the best fitting curves are shown in Fig. 2.

Several interesting conclusions follow from the estimated values. First, we find that there is little consistency between parameter estimates obtained by different methods. Unfortunately, we do not know the real parameters (that was not the case in the previous example where data have been generated from a numerical solution of the basic model), and therefore there is no definite way to find which estimates are the correct ones. For example, there is no statistical difference between most estimates for α^+ and α^-, but the average selection coefficient (found by Method 2) is different from both boundary values (see Table 2).

Despite this unfortunate observation, there are trends consistent with all the methods used. First, we find that higher levels of plasmid gene expression have led to a higher cost of the plasmid (glycerol vs. glucose). This is, in turn, unsurprising since it is known that effective expression of plasmid genes may lead to lower stability plasmid-bearing cells (Nguyen *et al.*, 1989; Bentley *et al.*, 1990; Lenski *et al.*, 1994b). The estimated cost of the plasmid varies from 0.30 to 0.50 at low levels of plasmid gene expression and from 0.54 to 0.80 at high expression levels (in each particular case the difference is statistically significant).

Surprisingly, we find a different trend for the probability of plasmid loss τ_0: at high levels of plasmid gene expression τ_0 is low and vice versa (the order of magnitude is $\sim 10^{-3}$). It is not quite clear why this is case; there are many factors, however, which may influence τ_0 in different directions. For instance, high levels of plasmid

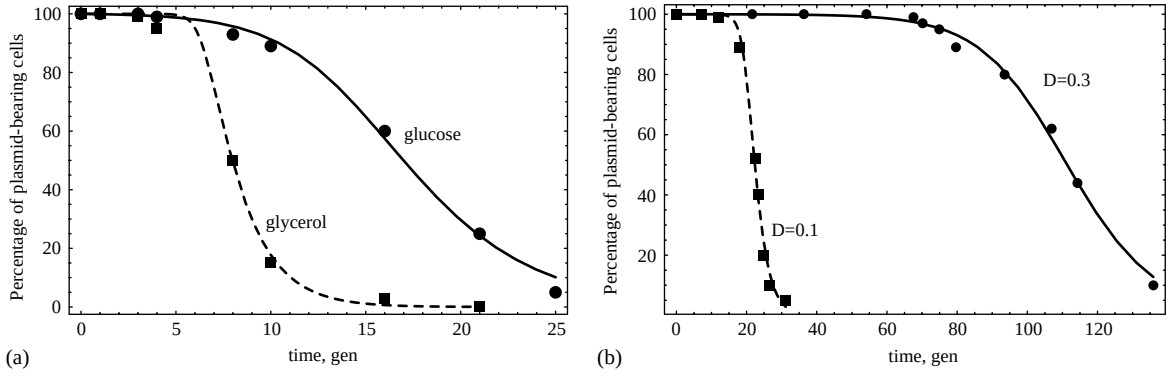

FIG. 2. The dynamics of plasmid-bearing cells in chemostat: experimental data. Panel A: the growth of bacteria with a plasmid pPHL-7 has been limited by different sugars, glucose and glycerol, at $D = 0.1\,\mathrm{hr}^{-1}$ (Popova *et al.*, 1992). Panel B: plasmid-bearing cells (pBR322) have grown at different dilution rates, $D = 0.1\,\mathrm{hr}^{-1}$ and $D = 0.3\,\mathrm{hr}^{-1}$ (Wouters *et al.*, 1980). "…": experimental data, "—": the best fit of the data by Method 2 [eqn (17)]. Parameters for the fitting are given in Table 2.

TABLE 2
Estimation of plasmid instability parameters: experimental data

Specific	Method 1*		Method 2†		Method 3‡		
condition	α	τ_0	α	τ_0	α^+	α^-	τ_0
Glucose§	0.30 ± 0.01	$(2.33 \pm 0.52) \times 10^{-3}$	0.36 ± 0.01	$(1.26 \pm 0.14) \times 10^{-3}$	0.53 ± 0.07	0.53 ± 0.06	$(0.57 \pm 0.19) \times 10^{-3}$
Glycerol	0.54 ± 0.02	$(1.65 \pm 0.73) \times 10^{-3}$	0.80 ± 0.01	$(0.55 \pm 0.51) \times 10^{-4}$	0.72 ± 0.01	0.58 ± 0.17	$(0.13 \pm 0.03) \times 10^{-3}$
$D = 0.1\,\mathrm{hr}^{-1}$‖	0.43 ± 0.02	$(0.63 \pm 0.54) \times 10^{-5}$	0.52 ± 0.01	$(1.73 \pm 0.43) \times 10^{-7}$	0.46 ± 0.01	0.44 ± 0.11	$(2.14 \pm 0.87) \times 10^{-5}$
$D = 0.3\,\mathrm{hr}^{-1}$	0.11 ± 0.05	$(1.30 \pm 0.61) \times 10^{-5}$	0.11 ± 0.01	$(1.05 \pm 0.01) \times 10^{-5}$	0.21 ± 0.02	0.13 ± 0.01	$(1.03 \pm 0.67) \times 10^{-7}$

*† Fitting by analytical solutions (14) and (17), respectively.
‡ Fitting of init ial and last points by solutions (12) and (11), respectively.
§ Popova *et al.* (1992).
‖ Wouters *et al.* (1980).

gene expression may influence the efficacy of replication and segregation of plasmids (such as to increase τ_0). In contrast, different substrates even at a fixed dilution rate may also affect the average copy number in the population and the distribution of cells with different plasmid copy number leading to changes in τ_0.

In addition, we find that for all used methods selection coefficient of bacteria, bearing plasmid pBR322, depends on the dilution rate in chemostat such as at low D α is higher and vice versa (Wouters *et al.*, 1980). This again is not a new result, even though previous estimates have been obtained using not completely verified methods [Method 1 and its derivatives (Caulcott *et al.*, 1987; Davidson *et al.*, 1990; Dunn *et al.*, 1995)]. The cost of the plasmid pBR322 in these conditions varies from 0.10 to 0.40. In contrast, the dependence of the probability of plasmid loss τ_0 on the dilution rate D is different for Methods 2 and 3. However, since $\alpha^+ \approx \alpha^-$ at $D = 0.1\,\mathrm{hr}^{-1}$ and $\alpha^+ \neq \alpha^-$ at $D = 0.3\,\mathrm{hr}^{-1}$, we conclude that τ_0 according to these data does not depend on the dilution rate in chemostat and is $\approx 1.7 \times 10^{-7}$.

Discussion

In this paper, we developed and analysed analytical methods for the estimation of parameters characterizing population instability of natural and recombinant plasmids during growth of plasmid-bearing cells in chemostat. By analysing the dynamics of bacteria according to the well established yet simple model (1), we found that the dynamics of plasmid-bearing cells and the limiting growth substrate in chemostat may be similar when selection coefficient is dependent on or independent of the substrate concentration (i.e. when $K_S^+ \neq K_S^-$ and $K_S^+ = K_S^-$, respectively, see Fig. 1). Because of that even in chemostat culture where environmental conditions for the bacterial growth are fixed (compare, for instance, with batch culture where many parameters change as the population grows), the estimation of instability parameters of plasmid-bearing cells, which are selection coefficient α and a probability of plasmid loss τ_0, is not simple.

By fitting the original dynamics of plasmid-bearing cells obtained by a numerical solution of the basic model (1), we found that (i) previously suggested methods for the estimation of the instability parameters give incorrect estimates for α and τ_0 due to making invalid assumptions regarding bacterial growth in chemostat (Cooper *et al.*, 1987; Caulcott *et al.*, 1987; Brilkov *et al.*, 1990; Davidson *et al.*, 1990; Dunn *et al.*, 1995); (ii) for the case when $K_S^+ = K_S^-$, a nonlinear solution (17) gives the most precise estimates for the average selection coefficient α and a probability of plasmid loss τ_0; (iii) when $K_S^+ \neq K_S^-$ neither of the methods using an approximation $\alpha = const$ could be used; instead by assuming that the substrate concentration is constant at the beginning and the end of cultivation [eqns (4) and (5)] the most reliable estimates for α^+ (selection coefficient, when most of cells in the population are plasmid-bearing), α^- (selection coefficient when most of cells in the population are plasmid-free), and τ_0 are found according to approximations (11) and (12).

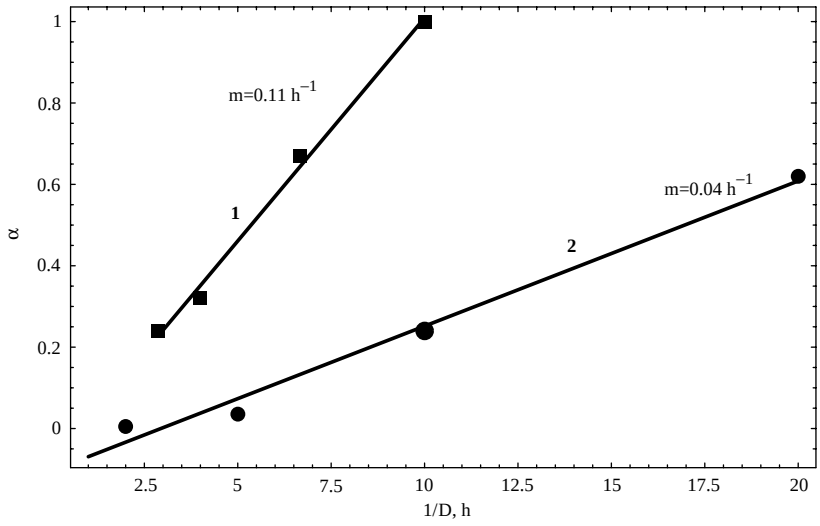

FIG. 3. The relationship between selection coefficient α and the dilution rate in chemostat D at which α was measured. "…": experimental data from (1) Godwin and Slater (1979) and (2) Duetz and van Andel (1991); "—": fitting by a linear regression model assuming $\alpha = m/D$. Interceptions for both regression curves are insignificantly different from zero; estimated expenditure for plasmid maintenance m is shown (see text for details).

Obviously, if the instability parameters are being estimated from real experimental data, it is not known *a priori* whether selection coefficient is constant in given environmental conditions (such as the limiting growth substrate, dilution rate in chemostat, etc.). Therefore, Method 3, which makes no assumption regarding constancy of the selection coefficient, should be used to estimate α^+, α^-, and τ_0. If the hypothesis $\alpha^+ \neq \alpha^-$ is not supported, the average selection coefficient and the probability of plasmid loss can be determined using Method 2 (see previous section for details).

One interesting and very important insight follows from the estimates for the selection coefficient of plasmid-bearing cells in chemostat at different dilutions (see Table 2‖). The fact that selection coefficient of plasmid-bearing cells may depend on the dilution rate in chemostat directly implies that selection coefficient is not constant and depends on the environment in which plasmid-bearing cells are grown. Non-constancy of α means that half-saturation constants for plasmid-bearing and plasmid-free cells are distinct because if $K_S^- = K_S^+$, then selection coefficient is independent of the dilution rate in chemostat (or substrate concentration) and is determined only by the maximum specific growth rates of plasmid-bearing and plasmid-free cells, $\alpha = 1 - \mu^+_{max}/\mu^-_{max} = const$.

This result also raises a question of whether the growth rate dependence of the population of plasmid-bearing cells on the limiting growth substrate can be described by a simple Monod function [given by eqn (2)] proven to be satisfactory for describing the growth of plasmid-free cells in chemostat with one limiting growth substrate (Pirt, 1975; Lendenmann *et al.*, 2000). One lucky guess might be to assume that the presence of a plasmid recruits some cell resources in a permanent manner such as the growth rate of plasmid-bearing cells is reduced by a constant amount: $\mu^+(S) = \mu^-(S) - m$. Then the cost of the plasmid is simply $\alpha = m/\mu^- \approx m/D$ [the last approximation is true if selection coefficient is measured at the end of cultivation where $\mu^-(S) \approx D$]. Surprisingly, we have found data where a relationship between the selection coefficient α and the reciprocal of the dilution rate in chemostat is linear (Godwin & Slater, 1979; Duetz & van Andel, 1991, see Fig. 3). Why some plasmids utilize a constant amount of cell resources (instead of, for instance, a constant fraction) regarding the energetic status of the host cell is not clear and requires future investigation.

PROBLEMS WITH THE SIMPLE MODEL

Using a simple mass-action model for the dynamics of plasmid-bearing cells in fixed environmental conditions in chemostat (1) and its analytical approximations, we can estimate two basic parameters governing the loss of plasmids during cultivation: selection coefficient α and the probability of plasmid loss τ_0. However, there are some experimental observations which are in contrast with such a simple model and which, therefore, can undermine our ability to estimate α and τ_0.

(i) The average copy number of a plasmid in the plasmid-bearing cell population may change while bacteria are grown in chemostat (Jones *et al*., 1980; Brownlie *et al.,* 1990; Brendel & Perelson, 1993). This in turn will change parameters α and τ_0 which obviously should depend on the plasmid copy number. In this case, models that account for changes in the plasmid copy number can be of particular use (Paulsson & Ehrenberg, 1998; Ganusov *et al*., 1999, 2000).

(ii) A copy number control of most plasmids is sloppy, i.e. the number of plasmid copies per daughter cell after cell division varies; therefore, in a population of plasmid-bearing cells there is always a distribution of cells with a different number of plasmid copies (Nordstrom *et al*., 1984; Ayala-Sanmartin & Gomez-Eichelmann, 1989; Lobner-Olesen, 1999). Hence, more realistic models of the population dynamics of plasmid-bearing cells should describe how a distribution of cells with a different plasmid copy number changes with time. Such models have already been suggested for the analysis of plasmid stability from the within-cell dynamics of plasmids (Paulsson & Ehrenberg, 1998) and population dynamics of plasmid-bearing cells with a different plasmid copy number (Bentley & Kompala, 1989; Bentley & Quiroga, 1993; Ganusov *et al*., 1999,2000,2001).

(iii) Finally, long-term maintenance of plasmid-bearing cells in chemostat may lead to coevolution of the plasmid and the host cell reducing as the cost of the plasmid (selection coefficient) as well as the probability of plasmid loss (Bouma & Lenski, 1988; Fleming *et al*., 1988; Impoolsup *et al*., 1989; Lenski *et al*., 1994a; Seegers *et al*., 1995).

Conclusion

Even at present time we do not completely understand why and how plasmids are lost during prolonged cultivation. The exact mechanisms by which plasmid-bearing cells grow at a slower rate than their plasmid-free counterparts are also unclear whether it is a direct effect of plasmid gene expression on the viability of plasmid-bearing cells, inhibition of the cell growth, or the use of cell resources on plasmid maintenance. Estimation of the plasmid instability parameters according to the methods developed in this paper is the first step in understanding the major causes of why plasmids are lost in culture or not lost in nature.

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